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Mechanisms of cellular cholesterol compartmentalization: recent insights

Elina Ikonen[1,2]

This review discusses advances in understanding how the controlled delivery of cholesterol between subcellular compartments is achieved and what novel experimental strategies are being employed to address this fundamental question. Recent work has focused on cholesterol-binding proteins that can facilitate directional cholesterol transfer between contacts of the ER and Golgi or late endosomal membranes. Increasing structural information on cholesterol-binding proteins, new modules engineered from them as well as improved imaging and gene editing techniques are providing valuable insights. There is also mounting information on how the crosstalk between cholesterol transport and nutrient signaling is orchestrated and how cellular fatty acid metabolism and cholesterol homeostasis are intertwined.

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Introduction

Cholesterol is an essential component of mammalian cell membranes. It is unevenly distributed between membranes (Figure 1) and rapidly exchanged between them. This article aims to briefly summarize interesting observations made during the past couple of years in the field of cellular cholesterol trafficking and homeostasis, and to point towards emerging trends. Increasing evidence suggests that besides membrane transport, lipid transfer proteins provide specific paths of cholesterol exchange between closely apposed membranes. Advances have been made, for example, in understanding late endosomal cholesterol transport, ER-to-Golgi sterol delivery and links between cholesterol transport, nutrient signaling and fatty acid metabolism. Moreover, improved tools such as biosensors deriving from cholesterol-binding protein modules have been employed for probing cholesterol compartmentalization. For further information on the topic, the reader is referred to more comprehensive reviews [1–5].

Late endosomal cholesterol binding proteins: the usual suspects in cholesterol transport

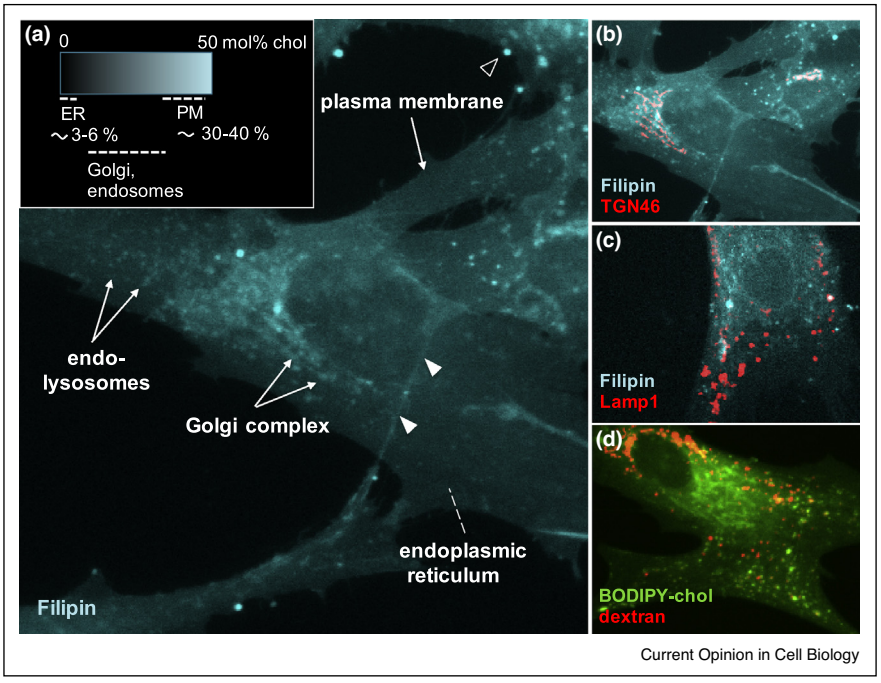
A major route of cholesterol entry into mammalian cells is via endocytosis of low density lipoproteins (LDL). Upon release from the LDL receptor and acid hydrolysis of the particle contents, the released cholesterol is removed from late endosomal organelles via the co-ordinated action of Niemann–Pick type C (NPC) 1 (NPC1) and NPC2 proteins. During the past few years, increasing structural information on NPC1 has revealed insights into the architecture of this complex protein with 13 membrane spans, including luminal interaction with NPC2 and Ebola virus [6–8], membrane embedded sterol-sensing domain [9] and C-terminal luminal domain [10].

Despite these developments, how cholesterol reaches the lysosomal limiting membrane remains mysterious. Cholesterol somehow traverses the ~8 nm thick lysosomal glycocalyx and the heavily glycosylated, abundant lysosomal membrane proteins LAMPs that can also bind cholesterol, play a role [11,12]. Indeed, cholesterol can bind to protein domains located at a considerable distance (12 Å) from the membrane spanning region, as shown, for example, for the extracellular domain of Smoothened [13]. However, also in this case the mechanism by which cholesterol gains access to this binding pocket is unknown.

From lysosomes, LDL-derived cholesterol is delivered to other membrane compartments. Recent findings argue that the bulk of LDL-cholesterol is transported to the plasma membrane, to replenish this cholesterol pool(s) [14,15]. One of the regulators of this route is Rab8a that is recruited in an NPC1-dependent manner to cholesterol-enriched late endosomal organelles and promotes their delivery to the leading edge of the cell [16]. Remarkably, in the absence of functional NPC1, the therapeutic drug candidate β-cyclodextrin can induce subplasmalemmal redistribution of the storage lysosomes and their subsequent release to the extracellular space [17].

The mechanisms of post-NPC1 cholesterol trafficking to the ER remain puzzling, despite the tantalizing presence of cytoplasmically oriented cholesterol-binding and ER interacting domains in late endosomal membrane

Figure 1



Subcellular compartmentalization of cholesterol. Primary human fibroblasts were grown in complete medium and sterol detected by filipin or BODIPY-cholesterol. **(a)** Cholesterol is enriched in the plasma membrane, where it represents about 30–40 mol% of lipids. Plasma membrane protrusions are often cholesterol enriched. Please note a tunneling nanotube connecting two cells (white arrowheads) and plasma membrane derived vesicles/blebs, typical for fibroblast cultures (empty arrowhead). The ER extends throughout the cell but its cholesterol content is low (typically 3–6 mol% of lipids) and not visualized by filipin (dotted line). The cholesterol content of the Golgi complex is intermediate between ER and plasma membrane, increasing in cis-to-trans direction. **(b)** Overlay of filipin staining in A with anti-TGN46 antibody staining. The cholesterol content of endo-lysosomal organelles varies considerably pending on incoming lipoprotein cholesterol uptake. **(c)** Overlay of filipin and anti-Lamp1 staining. **(d)** Cells labeled overnight with dextran and BODIPY-cholesterol. Please note BODIPY-cholesterol perinuclear Golgi-like labeling and partial colocalization with dextran-positive lysosomes.
Source: Images courtesy of Maarit Hölttä-Vuori, stainings and imaging performed as in Refs. [20,37].

proteins [3,4]. In particular, the ORP1L and STARD3 proteins can bind VAP in the ER, bridging between late endosomes and the ER. Interestingly, recent reports provide evidence that both ORP1L and STARD3 transfer cholesterol to the opposite direction, that is, from the ER to endosomes, employing VAP as the ER partner [18•,19•]. STARD3 expression induces cholesterol accumulation in endosomes and favours the formation of pleomorphic membrane structures inside endosomes [19•]. This is in line with earlier findings that dehydroergosterol (DHE) accumulates in late endosomes expressing STARD3 [20].

The ORP1L–VAP interaction is needed for ER-endosome cholesterol transport under low-cholesterol conditions, to support intraluminal vesicle formation in the endosome lumen [18•]. Accordingly, multivesicular body formation was reported to depend on cholesterol and ORP1L [21]. Furthermore, ORP1L–VAP interactions can establish contacts between the ER and late autophagosomes under low-cholesterol conditions [22]. These data fit with the idea that ORP1L–VAP affinity is increased when ORP1L is not sterol bound [23–25]. However, this is not as easy to reconcile with a model where ORP1L mediates cholesterol egress from the endo-lysosomal system under the high endosomal cholesterol conditions induced by LDL loading [26].

Nevertheless, under some conditions ORP1L facilitates cholesterol transfer towards the ER. Upon acute adenoviral infection the viral RIDα protein hijacks the ORP1L sterol-binding domain and apparently induces a novel trafficking route [27]. On the whole, the specialized roles of vesicle subpopulations [28] and dynamic communication between membranes that influences endosome motility, fission, and cargo trafficking are becoming increasingly apparent [29]. In this scenario proteins may not only be recycled but also repurposed, pending on the metabolic cues and local co-incidence of other machinery components.

Role of OSBP in ER–Golgi cholesterol transport

The ER receives cholesterol from the plasma membrane, other endomembranes and via *de novo* synthesis. Yet, the ER cholesterol content is kept low (normally ~a few mol % of lipids, Figure 1), necessitating efficient cholesterol export. One of the proteins topologically suited for this task is OSBP with its pH domain binding PI4P in the TGN, FFAT motif binding VAP in the ER and ORD domain capable of binding and transferring cholesterol. This model, first assessed *in vitro* with purified components [30], was recently verified in mammalian cells, taking advantage of the compound OSW-1 to rapidly switch off endogenous OSBP [31••]. A 2-h treatment with the drug was accompanied by reduced DHE labeling in the TGN and increased labeling in lipid droplets, suggesting altered ratio between forward and backward sterol movement between the late and early secretory pathway.

PI4P in the TGN was consumed by OSBP via exchange against sterol and thus necessitating continuous replenishment by PI4-kinase. Interestingly, the ER-TGN contact sites were apparently driven by the kinase and were highly dynamic, moving pending on enzyme activity, with OSBP following. Notably, a 3-day silencing of OSBP resulted in DHE redistribution from the TGN not to lipid droplets but to late endosomal structures, speaking for some compensatory effects manifesting at longer time points [31••]. This is important to keep in mind when chronically depleting components from well safeguarded machineries. At least in this case, a few days seemed sufficient to induce such effects.

Widening perspectives: cholesterol transport, nutrient signaling and fatty acid metabolism

There are also increasing insights into the interplay between cholesterol homeostasis and nutrient signaling via the master nutrient sensor mTORC1. Castellano *et al.* studied the effects of cholesterol at the level of late endosomal organelles and provided evidence for a dual regulation of mTORC1 via LDL-derived cholesterol [32••]. Cholesterol activates mTORC1 via interaction with the putative amino acid transporter SLC38A9. On the other hand, NPC1 interacts with SLC38A9 and mediates mTORC1 inhibition under cholesterol starvation conditions. This activity of NPC1 is abolished in the sterol-sensing domain mutant NPC1-P691S, implicating that it is somehow linked to the sterol sensing/transfer function of NPC1.

In another line of investigation, Zha and coworkers found that mTORC1 activity is required to maintain endosomal recycling and to prevent lysosomal delivery of recycling receptors and membrane lipids [33•]. Interestingly, mTORC1 also controls SREBP-2 via this membrane routing: as high mTORC1 activity maintains endosomal recycling and suppresses autophagy, less (recycling and autophagosomal) membrane-derived cholesterol is trafficking to lysosomes. This, in turn, keeps ER cholesterol low and SREBP-2 active. An intriguing issue is why the increased flow of cholesterol to the plasma membrane is not reflected as increased cholesterol delivery to the ER and consequent inactivation of SREBP-2. This could be related to the high potential of the plasma membrane to sequester cholesterol [34] and/or to the analysis time point after refeeding.

Recent elegant work using *in vivo* models highlights how cholesterol compartmentalization is critically affected by other membrane lipids. Endogenous fatty acid synthesis in macrophages plays an important role in controlling cellular cholesterol distribution and metabolism. In fatty acid synthase (FAS) deficient macrophages, the cholesterol content was decreased and cholesterol was more readily released from the plasma membrane [35••]. Moreover, the production of endogenous fatty acids was necessary for the assembly of cholesterol-dependent inflammatory signaling networks at the plasma membrane, modulating inflammation and insulin resistance at the whole-body level.

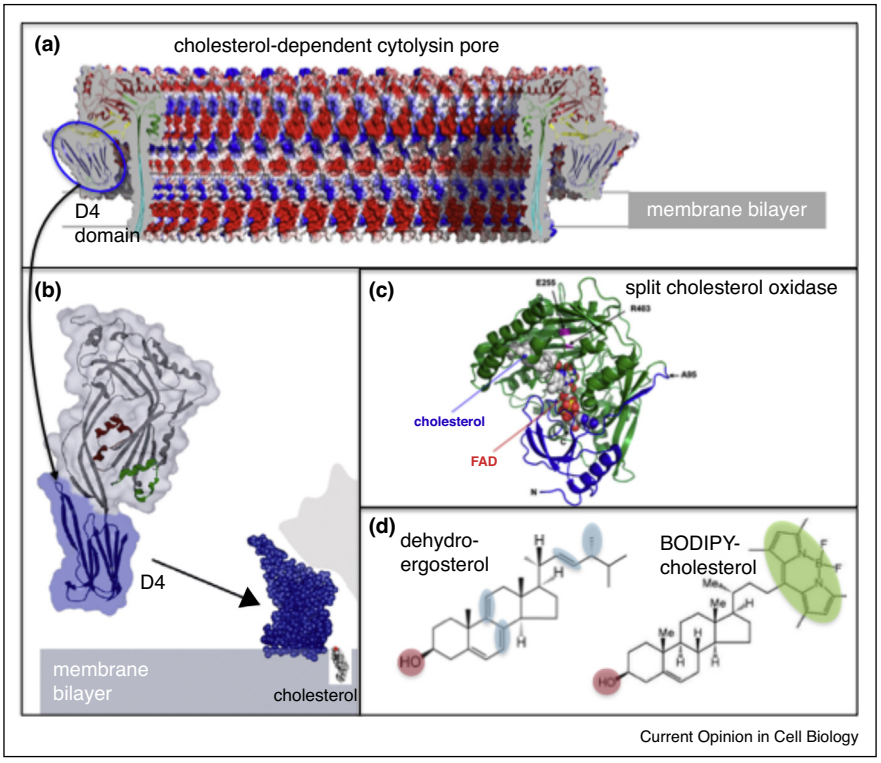
Mechanistically, the effect of fatty acids may be mediated, for example, via the acyl chain composition of phospholipids. For instance, the structure of phosphatidylcholine has been shown to influence SREBP-1 processing via the SCAP pathway. The phospholipid remodelling enzyme LPCAT3 drives the incorporation of polyunsaturated fatty acids in the ER, and by promoting the generation of PC with 2 or 4 double bonds in the fatty acids (linoleyl or arachidonoyl PC), stimulates SREBP-1 maturation [36].

Sharpening tools for cholesterol detection

Optimally, cholesterol trafficking would be visualized conveniently by imaging at high subcellular resolution. With the current tools, this remains a challenge. Both the fluorescent sterols and sterol-binding protein domains employed for the purpose have caveats, but there is increasing awareness of their applications/limitations, and improvements have been made.

As an example, in the case of BODIPY-cholesterol (TopFluor-Cholesterol) both the method of delivery and analysis time point affect the distribution. When administered from solvent, sterol sequestration characteristic of a lysosomal storage disease becomes evident only after equilibration with membranes [37]. DHE that is structurally more similar to cholesterol (Figure 2), has suboptimal fluorescence properties (UV excitation, rapid bleaching). Yet, improved optics and cameras enabled more accurate measurements, leading to the re-estimation of the rate of sterol transport between the endocytic recycling compartment and the plasma membrane (now

Figure 2



Select cholesterol binding proteins and fluorescent sterols used for studying cholesterol compartmentalization. **(a)** Bacterial cholesterol-dependent cytolysins kill cells by binding to the 3-OH groups of cholesterol molecules and oligomerizing to generate a membrane pore. Cryo-electron tomographic structure of pneumolysin (adapted from Ref. [51]). **(b)** The juxtamembrane D4 domain binds cholesterol, shown for perfringolysin O (adapted from Ref. [52]). **(c)** Schematic structure of the inducible split cholesterol oxidase, with the N-terminal fragment in blue and C-terminal fragment in green (from [46]). **(d)** Structures of two fluorescent sterol analogs. The differences to cholesterol are indicated with blue (dehydroergosterol) or green (BODIPY-cholesterol, a.k.a. TopFluor-cholesterol).

measured $t_{1/2}$ = 12–15 min) and the conclusion that STARD4 accounts for 1/4 of this transport [38]. Interestingly, a 20% increase in cellular cholesterol content had a major kinetic effect, increasing the DHE transport rate 3–4-fold.

The domain 4 (D4) of the bacterial toxin perfringolysin O (PFO) or anthrolysin O (ALO) has been developed as a cholesterol-biosensor. D4 is a 13 kDa protein that recognizes the 3-OH group of cholesterol when present at 30 mol% or higher (Figure 2). The higher affinity variant D4H has a slightly lower threshold for cholesterol [39]. Importantly, a recent study characterized additional point mutants of PFO D4, including mutants that can recognize a range of cholesterol concentrations to as low as 1 mol% [40••]. These fluorescently labeled biosensors were applied extracellularly or to the cytoplasm (via microinjection) into commonly used cell types and membrane bindings were recorded.

Based on the results, the exofacial leaflets were estimated to contain 30–40 mol% and cytosolic leaflets 2–5 mol% of cholesterol. Interestingly, ABCA1 and ABCG1 were shown to maintain the low cholesterol concentration in the inner plasma membrane leaflet, whereas knockdown of NPC1, ORP5 or STARD4 did not affect the transbilayer distribution of cholesterol [40••]. However, it is worth noting that the transbilayer distribution of cholesterol remains controversial. According to other estimates, cholesterol may rather be enriched in the inner leaflet [41] or be homogeneously distributed between leaflets [42]. Although no probes come without pitfalls, the strategy and observations by Liu *et al.* [40••] represent the most sophisticated assessment of cholesterol transbilayer distribution so far. With high spatial imaging resolution, these sterol-binding probes may also give insights into the lateral distribution of cholesterol. For instance, nanoscale secondary ion mass spectrometric imaging of D4 domains demonstrated that the accessible cholesterol was highly enriched in microvilli [43].

Indeed, cholesterol-binding protein domains bind to the accessible fraction of cholesterol, that is, the fraction that is capable of interacting with water-soluble molecules at the surface and is not shielded due to associations with neighboring lipids (see e.g. [44]). Another obvious caveat

of these domains is that they sequester cholesterol and may modify its partitioning in the membrane and between membranes. Infante and Radhakrishnan took advantage of this behaviour of ALO D4, employing it as an inhibitor of cholesterol transport from the plasma membrane to the ER. Using this approach, they concluded that LDL-cholesterol moves initially from lysosomes to the PM, and subsequently to the ER to suppress activation of SREBPs [15]. It has also been noted that D4 binding is not necessarily a suitable measure of the membrane cholesterol content, especially when comparing different kinds of cells or membranes. For example, D4 cholesterol accessibility in human erythrocytes varied over 10-fold among individuals and this was unrelated to the membrane cholesterol content but rather associated with the phospholipid composition [45].

Another bacteria-derived tool in cholesterol research is cholesterol oxidase that hydrolyzes the 3-OH group to produce cholestenone. One of the issues hampering its cell biological use is high efficiency and thereby toxicity. To improve the controllability and tracking of the enzyme, a split, rapamycin-inducible fluorescent cholesterol oxidase was recently engineered [46] (Figure 2). When the split halves are introduced into human cells, rapamycin allows temporal control of the enzyme activity, with initiation of oxidation upon addition of the drug and loss of enzyme expression with concomitant recovery of cholesterol levels upon its removal.

Concluding remarks

Advances in the field of cholesterol trafficking are not effortless, due to the complexity of lipid metabolic regulation, but are stimulated by the privilege of standing on the shoulders of giants (see e.g. [5]). The ongoing development of novel tools and experimental strategies provide incentives to keep up with the high standards. Progress is to be expected for instance in structure–function relationships of cholesterol-interacting proteins and in harnessing lipid transport and metabolism by editing endogenous cellular proteins.

As exemplified in the case of NPC1 protein, the upsurge in protein structural analysis by X-ray crystallography and cryo-electron microscopy is starting to reveal atomic-level insights into how cholesterol interacts with proteins and influences their behaviour. Moreover, important proteins controlling cholesterol transport and distribution in mammalian cells most probably still remain to be characterized, as suggested by the identification of mammalian proteins in the LAM (lipid transfer proteins anchored at membrane contact sites) family that contain StART-like lipid transfer domains and are capable of transferring cholesterol [47,48].

In the cellular context, robust overexpression or chronic knockout of a protein is rarely an optimal strategy, especially when a protein is part of a large interlinked network, as in lipid homeostatic regulation. For instance, it has been known for 10 years that the cholesterol threshold of 5 mol% ER lipids — that inhibits SREBP-2 export from the ER — becomes 3 mol% when cells express elevated amounts of Insig-1 [49]. Besides shifting the balance between protein functions, persistent protein manipulation often leads to compensatory changes that abolish or mitigate the primary perturbation.

CRISPR/Cas9 based gene editing has introduced a major paradigm shift in this area. It is now feasible to study proteins expressed from their endogenous loci, introducing tags that facilitate rapid re-localization in cells, targeting for degradation, affinity capture, and so on — pending that the tag does not perturb the protein function. Moreover, increasingly elegant screens utilizing CRISPR/Cas9 based approaches are within reach, as shown, for example, by the recent identification of a novel E3 ligase regulating HMGCR degradation and cholesterol biosynthesis [50]. Together with the ongoing development of cholesterol detection methods and structural information on protein–lipid interactions, these tools are helping to reveal novel players in cholesterol compartmentalization.

Conflict of interest statement

Nothing declared.

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